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Kinematic Changes During the Cryopreservation of Boar Spermatozoa

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ABSTRACT: The present study evaluates the effect that various steps of a conventional cycle of cryopreservation have on the patterns of movement exhibited by boar spermatozoa. Sperm-rich ejaculate fractions collected from 24 mature fertile boars (1 ejaculate per boar) were cryopreserved following a standard freeze-thaw procedure with 0.5-mL plastic straws. Overall sperm motility and the individual kinematic parameters of motile spermatozoa (assessed by the computer-aided sperm analysis system Sperm Class Analyzer [SCA]) were recorded in 5 steps of the cryopreservation procedure. These steps were as follows: 1) at the time that the fresh semen was extended, 2) at 17°C, after sperm concentration by centrifugation and re-extension of the pellet with lactose–egg yolk extender; 3) at 5°C, after added freezing extender; 4) at the time that thawed semen was held in a water bath at 37°C for 30 minutes; and 5) at the time that thawed semen was held in a water bath at 37°C for 150 minutes. Data from individual motile spermatozoa, defined by 7 kinematic parameters (curvilinear velocity [VCL], straight-line velocity [VSL], average path velocity [VAP], linearity [LIN], straightness [STR], mean amplitude of lateral head displacement [ALH], and beat cross frequency [BCF]), were analyzed using a pattern analysis technique (PATN) to identify and quantify populations and subpopulations of motile sperm within the semen samples. After the first cluster analysis, 3 motile sperm populations (P) were identified (P1: progressive and/or vigorous cells [90.4%], P2: poorly progressive cells [8.3%], and P3: nonprogressive cells [1.3%]). These populations remained constant ($P > .05$) throughout the 5-step cryopreservation procedure. A second PATN was carried out within the P1 sperm population, which identified 3 sperm subpopulations (sP) (eg, sP1: cells with progressive and vigorous movement [58.7%], sP2: progressive cells only [24.6%], and sP3: vigorous cells only, hyperactive-like [16.7%]). Although the relative frequency of these 3 subpopulations varied among ejaculates (boars), there was no interaction with any cryopreservation step we examined. Whereas sP1 remained constant ($P > .05$), sP2 and sP3 varied significantly ($P < .05$) through the cryopreservation procedure, with the increase in sP3 after centrifugation at 17°C and during cooling at 5°C considered particularly relevant. In conclusion, the present study confirms the heterogeneity of sperm movement patterns in boar semen, patterns that vary through the cryopreservation procedure, especially after removal of the seminal plasma by centrifugation and subsequent extension at 17°C and after the slow cooling at 5°C, when obvious increases in hyperactivated movement appeared. The vast majority of spermatozoa, those exhibiting progressive and vigorous movement, remained constant during the cryopreservation procedure, although the proportion differed among boars.

Key words: Kinematic parameters, sperm subpopulations, pig.
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Frozen-thawed boar spermatozoa are not widely used in commercial artificial insemination (AI) programs, because current cryopreservation protocols do not provide fertility levels comparable to those achieved with fresh or cooled liquid semen (Johnson et al, 2000). Cryopreservation induces both a loss of sperm viability and an impairment of functionality of the spermatozoa surviving the final step, the thawing (Watson, 2000). As boar spermatozoa are particularly sensitive to chilling stress because of their membrane composition (White, 1993), no more than 50% of boar spermatozoa are capable of surviving the cryopreservation cycle, even under optimal conditions (Eriksson and Rodriguez-Martinez, 2000; Carvajal et al, 2004). Furthermore, the poor functionality of those spermatozoa that survive thawing is particularly apparent when deposited in the sow genital tract. When similar numbers of motile spermatozoa are used for insemination, the fertilizing ability of liquid semen is higher than that of frozen-thawed spermatozoa (Waberski et al, 1994). Many of the frozen-thawed spermatozoa show a shorter life span and have difficulties in reaching the oocytes and penetrating their vestments after conventional AI. Such impairment could be due to changes in motility

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patterns following semen handling during cryopreservation.

Sperm motility is important for some steps of sperm transport within the female reproductive tract, primarily, for the penetration of the zona pellucida. During conventional cryopreservation procedures, ejaculated boar spermatozoa undergo extension, centrifugation, cooling, exposure to cryoprotectant, freezing, and thawing. Each of these steps is potentially harmful to the functionality of the spermatozoa, and each step adversely affects the movement pattern of those spermatozoa surviving the process, since they exhibit more lineal and less vigorous motility immediately postthaw than do those examined during the cooling step (Eriksson et al, 2001). However, there is a lack of systematic studies that have the goal of examining differences on sperm movement patterns between liquid and frozen-thawed semen (Holt et al, 2005).

Since boar spermatozoa in an ejaculate constitute a heterogeneous population in terms of their patterns of movement (Abaigar et al, 1999), we have tested the hypothesis that spermatozoa differ in their pattern of movement, cluster building, and reaction during the procedure of cryopreservation, including thawing. To test this hypothesis, spermatozoa considered motile by a computer-assisted motility analyzer (CASA) were allotted to different sperm populations according to their individual kinematic parameters using a series of multivariate pattern cluster analyses (Abaigar et al, 1999); then, the progress of these sperm primary populations, as well as the subpopulations derived from the largest primary population was evaluated through the different steps of a conventional boar semen cryopreservation procedure.

Materials and Methods

Semen Processing and Sperm Cryopreservation

Twenty-four ejaculates collected from 24 healthy mature and fertile boars (1 ejaculate per boar) undergoing regular semen collection for commercial AI were used in this experiment. Sperm-rich ejaculate fractions were collected by the gloved hand method, extended (1:1, vol/vol) in Beltsville Thawing Solution (BTS), and shipped to the Laboratory of Andrology of the Veterinary Teaching Hospital of the University of Murcia, Spain, within 1 hour. At the laboratory, after assessing sperm quality, the ejaculates were cryopreserved following a standard medium-straw (0.5 mL) freezing procedure initially described by Westendorf et al (1975) and recently modified by Carvajal et al (2004). Extended sperm-rich fractions were slowly cooled to 17°C for 240 minutes. At this temperature, the extended sperm suspension was centrifuged at 2400 × *g* for 3 minutes in a refrigerated centrifuge (Megafuge 1.0 R, Heraeus, Hanau, Germany). The supernatants were discarded, and the pellets were reextended with a lactose–egg yolk (LEY) extender (80% vol/vol 310 mM β-lactose, 20% vol/vol egg yolk, 100 μg/mL kanamycin sulfate, pH 6.2, and 330 ± 5 mOsm kg^{-1}) to a concentration of 1500 × 10^6 cells/mL. After further cooling to 5°C and storage for 120 minutes, the extended spermatozoa were resuspended with LEY-Glycerol-OrvusESPaste (LEYGO) extender (92.5% LEY, 6% glycerol, and 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass] vol/vol, pH 6.2, and 1650 ± 15 mOsm kg^{-1}) to a final concentration of 1000 × 10^6 cells/mL. Then, the extended and cooled spermatozoa were packed into 0.5-mL polyvinyl chloride French straws (Minitüb, Tiefenbach, Germany), which were frozen horizontally using a programmable cell freezer (IceCube 1810, Minitüb) as follows: frozen from 5°C to −5°C at 6°C/min and from −5°C to −80°C at 40°C/min; held for 30 seconds at −80°C; cooled at 70°C/min to −150°C; and then plunged into liquid nitrogen (LN_2). After 1 week of storage in LN_2, some straws were thawed in a circulating water bath at 37°C for 20 seconds. Thawed spermatozoa from 2 straws were extended at 37°C in BTS (1:2, vol/vol), pooled, and incubated in the water bath for up to 150 minutes.

Sperm Motility Analysis

Equipment—The assessment of the various kinematic parameters of motile spermatozoa was carried out with a CASA system (Sperm Class Analyzer [SCA] 5.0, Microptic, Barcelona, Spain). The extended spermatozoa were loaded into a Makler cell chamber (Sefi Medical Instruments, Haifa, Israel), examined using a Labophot microscope with warmed stage (Nikon, Tokyo, Japan) equipped with a phase-contrast 10× PL objective, and recorded using a monochrome video camera (CCD Hitachi, Tokyo, Japan) linked via a 2.5× projection eyepiece.

Setup Procedure—The analyses of sperm motility patterns were performed with the SCA system operating at 25 video frames per second (25 Hz). Each individual sperm track was identified within 16 sequential video frames (corresponding to 0.6 seconds of movement). The search radius was 11.49 μm, and spermatozoa with an average velocity of less than 10 μm/s were considered immotile.

Assessment Procedure—Prior to the assessment of sperm movement, spermatozoa were re-extended with BTS to 15 to 20 × 10^6 cells/mL and were equilibrated for 5 minutes at 37°C. For each evaluation, a 4-μL loaded prewarmed Makler chamber was placed on the warm (39°C) microscope stage. Nine fields per sample were examined (3 drops per sample and 3 fields per drop), and a minimum of 200 spermatozoa were recorded per cryopreservation step per ejaculate. Before the track sequence was to be analyzed, the trajectory of each spermatozoon identified and recorded in each field was visually assessed to eliminate possible debris and to diminish the risk that unclear tracks were included in the analyses.

Kinematic Parameters—The kinematic values recorded for each spermatozoon included, in addition to the overall percentage of motile spermatozoa, the velocity of movement, the width of the sperm head's trajectory, and the frequency of the change in direction of the sperm head (Mortimer, 2000). The velocity values recorded were the curvilinear velocity (VCL, μm/s) (eg, the average path velocity of the sperm head along its actual trajectory), the straight-line velocity (VSL, μm/s) (eg, the average path velocity of the sperm head along a straight line from its first to its last position), the average path velocity (VAP, μm/s) (eg,

the average velocity of the sperm head along its average trajectory), the percentage of linearity (LIN, %) (eg, the ratio between VSL and VCL), and the percentage of straightness (STR, %) (eg, the ratio between VSL and VAP). The width of the sperm head's trajectory value recorded was the mean amplitude of lateral head displacement (ALH, μm), the average value of the extreme side-to-side movement of the sperm head in each beat cycle. Finally, the frequency of the change in direction of the sperm head was recorded by means of the beat cross frequency (BCF, Hz), the frequency with which the actual sperm trajectory crosses the average path trajectory. For more detailed descriptions of these parameters, see Davis and Siemers (1995) and Mortimer (1997).

Sperm Viability Assessment

Sperm viability was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity. These characteristics were analyzed simultaneously using a triple fluorophore stain procedure, adapted for boar spermatozoa by Carvajal et al (2004), which includes the deoxyribonucleic acid–specific fluorochrome propidium iodide (PI), the mitochondria-specific fluorochrome rhodamine-123 (R123), and the acrosome-specific fluorochrome fluorescein isothiocyanate–labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) from Sigma Chemical Co (St Louis, Mo). Two hundred spermatozoa were examined under 1000× magnification (Eclipse E800, Nikon) using a BV-2A filter (400- to 440-nm excitation filter, 455-nm emission, 470-nm barrier filter). Spermatozoa that showed only green fluorescence over their mid-piece (R123 positive) were considered viable with an intact acrosome. Values were expressed as percentages.

Experimental Design

The overall sperm motility and viability and the individual kinematic parameters of motile spermatozoa were recorded at the following 5 steps of a conventional boar semen cryopreservation procedure:

1) Immediately after arrival of the BTS-extended sperm-rich ejaculate fraction (22°C) at the Laboratory of Andrology (step 1).
2) After centrifugation and extension of the sperm pellet with LEY extender at 17°C (step 2).
3) After adding the LEYGO extender at 5°C (step 3).
4) After thawing and re-extension with BTS (1:1, vol/vol) and holding in a water-bath at 37°C for 30 minutes (step 4).
5) After holding the re-extended thawed semen in a water bath at 37°C for 150 minutes (step 5).

Statistical Analysis

Data from all spermatozoa in the analysis were merged into a single data set that represented 30 568 motile spermatozoa, each one defined by the 7 kinematic parameters obtained from the CASA analysis (SCA) referred to above. A multivariate pattern analysis was carried out using the pattern analysis statistical computer program (PATN; CSIRO, Canberra, Australia) to classify the spermatozoa of the data set into a reduced number of populations according to their patterns of movement as previously described (Abaigar et al, 1999). In a first cluster analysis, 3 sperm populations were defined and named P1 to P3. A second PATN cluster analysis was performed for the 27 637 motile spermatozoa was included in population 1 (P1), which was identified as composed of 3 subpopulations, which were named sP1 to sP3. The summary statistics of the relative frequencies of spermatozoa belonging to the different populations or subpopulations were calculated and compared by a 1-way analysis of variance (ANOVA) (SPSS, version 12.0, SPSS Inc, Chicago, Ill).

The influence of each cryopreservation step and ejaculate on the relative distribution frequency of spermatozoa belonging to each population or subpopulation was analyzed by a 2-way ANOVA, using a general linear model, after arcsine transformation of percentage data. Likewise, the overall percentages of motile and viable spermatozoa were analyzed by a 2-way ANOVA.

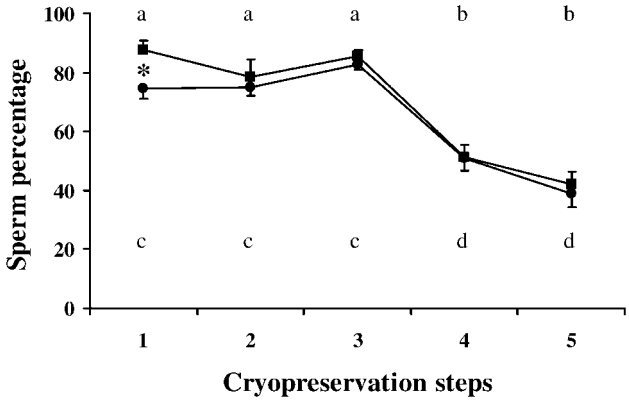

Figure 1. Changes in overall percentages of spermatozoa considered viable, with intact acrosome (■) and motile (●) through a cryopreservation cycle. **(a, b)** and **(c, d)** indicate differences between the cryopreservation steps for sperm viability or motility, respectively. * indicates differences between frequencies of viable and motile spermatozoa within the same cryopreservation step.

Results

Overall Sperm Motility and Viability

The mean percentages of motile and viable spermatozoa in the 24 ejaculates collected (1 ejaculate per boar) and examined throughout the 5-step cryopreservation procedure are shown in Figure 1. The cryopreservation stage influenced ($P < .01$) both sperm motility and viability. After thawing (steps 4 and 5), the percentages of motile and viable spermatozoa decreased ($P < .01$) when compared with prefreezing steps (steps 1, 2, and 3). In step 1, significant differences ($P < .01$) were observed between motile and viable spermatozoa, with the percentage of the latter higher than that of motile spermatozoa. In the other 4 steps (2 to 5), the percentages of motile and viable spermatozoa were similar ($P > .05$).

Motile Sperm Populations and Subpopulations

Three sperm populations were identified after the PATN analysis of the 30 568 individual motile spermatozoa.

Table 1. *Statistical descriptors of motility kinematics for the 3 boar sperm populations defined after pattern analysis (P1, P2, and P3)**

	Kinematic Parameters	Sperm Populations (P)		
		1	2	3
	Number (%)	27 637 (90.4) A‡	2529 (8.3) B	402 (1.3) C
VCL	Mean ± SEM	73.38 ± 0.32 A	36.23 ± 0.36 B	12.01 ± 0.32 C
	Median	73.01	35.61	10.99
	Percentiles†	42.52–98.64	26.68–45.37	8.01–14.60
VSL	Mean ± SEM	43.54 ± 0.22 A	5.59 ± 0.09 B	0
	Median	40.31	5.10	0
	Percentiles	18.45–64.11	3.8–7.66	0
VAP	Mean ± SEM	55.43 ± 0.25 A	15.86 ± 0.16 B	5.54 ± 0.21 C
	Median	55.04	15.05	5.19
	Percentiles	28.36–78.53	11.86–18.94	2.99–7.46
LIN	Mean ± SEM	63.09 ± 0.19 A	16.59 ± 0.25 B	0
	Median	67.59	17.03	0
	Percentiles	43.96–84.12	10.85–22.99	0
STR	Mean ± SEM	80.14 ± 0.17 A	35.88 ± 0.48 B	0
	Median	88.16	38.99	0
	Percentiles	71.18–96.21	26.56–48.96	0
ALH	Mean ± SEM	7.18 ± 0.05 A	5.65 ± 0.07 B	2.99 ± 0.09 C
	Median	4.89	4.93	2.81
	Percentiles	3.82–7.36	4.06–6.59	2.37–3.19
BCF	Mean ± SEM	11.68 ± 0.04 A	13.83 ± 0.23 B	0.34 ± 0.12 C
	Median	11.67	12.49	0
	Percentiles	8.33–15.01	8.33–18.33	0

* VCL indicates curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness, ALH, mean amplitude of lateral head displacement; and BCF, beat cross frequency.
† Percentiles: 25 and 75.
‡ Different letters indicate significant differences within rows ($P < .05$).

Summary statistics of these populations are shown in Table 1, and the qualitative interpretation of them is as outlined in the following paragraphs.

P1 contained the largest number of spermatozoa (90.4%) and depicted the most vigorous (highest VCL and ALH and high BCF) and/or progressive (highest VSL and VAP) movement, spermatozoa whose forward swimming line followed either rectilinear or parabolic tracks.

Population 2 (P2) included those spermatozoa whose movement was defined as less vigorous (low VCL and ALH), although they exhibited a high frequency of flagellar beat (the highest BCF), yielding 8.3% of the total motile population. They were poorly progressive (low STR and LIN), following irregular trajectories (the VCL being much higher that the VAP) and covering very short distances (very low VSL).

Population 3 (P3) contained the lowest number of spermatozoa (1.3%) and included those spermatozoa that moved their tails slowly (low BCF) and whose sperm heads had a low ALH, following a curved line (low VCL and very low VAP) without obvious forward motility (undetectable VSL, LIN, and STR).

The database of P1 (27 637 spermatozoa) was subjected to a second-cluster PATN analysis. Three different subpopulations (sP1–sP3) were identified as contributing to P1 (see Table 2). The majority (58.7%) belonged to sP1 and swam forward rapidly (high VSL) in a fairly straight line (high LIN and STR). The sperm trajectory was very regular and linear (their VAP was almost the same as their VCL), showing a moderate lateral head displacement (medium ALH).

The spermatozoa of sP2 (24.6%) were less vigorous (low VCL, ALH, and BCF) but, like those of sP1, drew very regular and linear trajectories (high values of LIN and STR). However, in contrast to sP1, the sP2 spermatozoa moved forward in slow motion, with little head lateral movement (the VAP was almost the same as the VCL) covering only short distances (low VSL).

Finally, sP3 (16.7%) appeared to be the most vigorous (high VCL, ALH, and BCF) but was not progressive (low VSL). The sperm trajectories were irregular (the VCL was much higher that the VAP, and the LIN was very low) and showed a high degree of lateral deviation of the head from the direction of movement (very low STR).

Frequency Distribution of Motile Spermatozoa Within the Different Populations and Subpopulations for Each Step of the 5-Step Cryopreservation Cycle

The proportion of motile spermatozoa within each of the 3 populations and 3 subpopulations was compared among ejaculates (boars). Ejaculate variability was identified for each of the 3 populations ($P < .01$) and subpopulations ($P < .01$). However, no significant interaction ($P > .05$)

Table 2. *Statistical descriptors of motility kinematics of the 3 boar sperm subpopulations (sP1, sP2, and sP3) obtained from the pattern analysis of the motile spermatozoa in the first population (P1)**

	Kinematic Parameters	Sperm Subpopulation (sP)		
		1	2	3
	Number (%)	16 222 (58.7) A‡	6808 (24.6) B	4607 (16.7) C
VCL	Mean ± SEM	82.35 ± 0.28 A	24.66 ± 0.21 B	113.95 ± 0.83 C
	Median	81.30	24.47	107.12
	Percentiles†	62.27–99.81	13.86–33.58	83.35–135.68
VSL	Mean ± SEM	60.92 ± 0.23 A	12.95 ± 0.10 B	26.58 ± 0.31 C
	Median	58.97	12.14	24.28
	Percentiles	43.57–75.52	7.79–17.61	14.92–35.33
VAP	Mean ± SEM	68.60 ± 0.25 A	15.90 ± 0.12 B	67.04 ± 0.57 C
	Median	67.78	15.56	62.88
	Percentiles	49.03–85.36	10.24–21.41	44.02–85.29
LIN	Mean ± SEM	74.56 ± 0.15 A	61.47 ± 0.39 B	24.08 ± 0.23 C
	Median	76.98	54.57	24.47
	Percentiles	63.97–86.54	40.72–84.87	14.09–33.99
STR	Mean ± SEM	89.33 ± 0.10 A	83.38 ± 0.22 B	42.09 ± 0.39 C
	Median	92.81	85.25	44.55
	Percentiles	84.52–97.12	73.14–96.31	25.53–57.29
ALH	Mean ± SEM	6.01 ± 0.04 A	3.77 ± 0.03 B	16.50 ± 0.19 C
	Median	4.89	3.67	13.91
	Percentiles	4.06–6.54	2.45–4.67	9.06–21.16
BCF	Mean ± SEM	12.70 ± 0.03 A	8.25 ± 0.08 B	13.11 ± 0.12 C
	Median	12.50	8.33	12.50
	Percentiles	10.42–15.00	3.85–11.67	9.37–15.39

* VCL indicates curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; ALH, mean amplitude of lateral head displacement; and BCF, beat cross frequency.
† Percentiles: 25 and 75.
‡ Different letters indicate significant differences within rows ($P < .05$).

was detected between the ejaculate and cryopreservation step.

P1 and P3 were always the largest ($P < .01$) and smallest ($P < .01$), respectively, within any given step of the 5-step cryopreservation procedure (Figure 2). The proportion of spermatozoa present in any of the 3 populations remained constant ($P > .05$) throughout the 5 steps.

The proportions of spermatozoa in each of the 3 sperm subpopulations identified within the motile spermatozoa of P1 are shown in Figure 3. Whereas sP1, the largest, remained constant ($P > .05$) throughout the 5-step cryopreservation cycle (Figure 3a), sP2 and sP3 varied. In detail, the proportions of spermatozoa within sP2 (Figure 3b) decreased ($P < .05$) from step 1 to steps 2 and 3 and then increased progressively ($P < .05$) after thawing (steps 4 and 5), the proportion of spermatozoa present in steps 1 and 5 remained similar ($P > .05$). sP3 (Figure 3c) showed an opposite trend to sP2, increasing ($P < .05$) from step 1 to steps 2 and 3 and then decreasing ($P < .05$) during the postthaw steps, with no changes observed during steps 1, 4, and 5 ($P > .05$).

Discussion

The process of cryopreservation, including freezing and thawing, induces structural and/or biochemical damage in boar spermatozoa, resulting in a drastic reduction of the percentages of viable and motile spermatozoa. As can be seen in Figure 1, while the percentages of total viable and motile spermatozoa remained high through the steps before freezing, they fell drastically after freezing and thawing, indicating that one or both of these steps can be lethal to a large number of spermatozoa. However, it is likely that a high proportion of those spermatozoa that die during the freezing and thawing steps undergo irreversible damage in the steps before freezing. In this context, slow cooling induces sublethal stress in boar sperm membranes (Watson, 2000).

The present study identified different motile sperm populations and subpopulations in boar ejaculates that underwent cryopreservation as determined by the combination of 7 CASA kinematic parameters. Several studies have tried to elucidate whether kinematic parameters in motile spermatozoa were useful for the prediction of in vivo or in vitro fertility, mainly in humans (De Geyter et al, 1998). The 7 kinematic parameters selected in the present study have been previously correlated elsewhere with in vivo fertility. A combination of 5 of these parameters from among VSL, VAP, VCL, LIN, ALH, STR, and BCF has proven useful in estimating the potential fertility for bulls (Farrell et al, 1998) and boars (Holt et al, 1997).

Initially, 3 different populations of motile spermatozoa

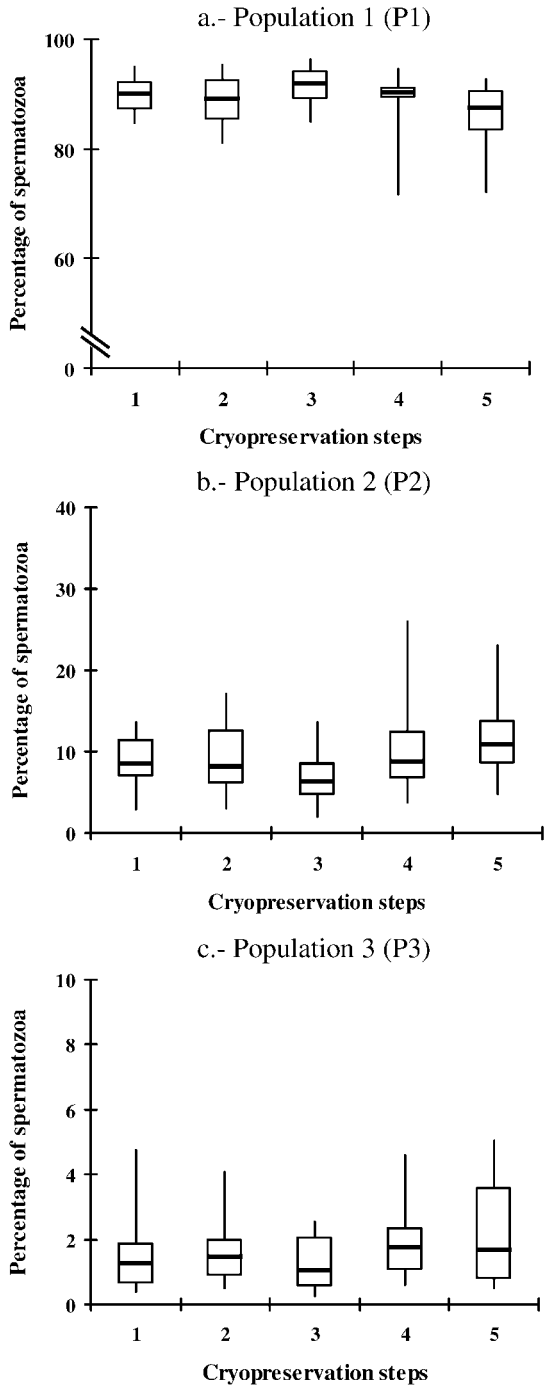

Figure 2. Whisker-box plots showing the frequency distribution for the 3 sperm populations distinguished by pattern analysis (P1–P3) within the motile spermatozoa throughout the 5 cryopreservation steps. Boxes enclosed the 25th and 75th percentiles, the dot is the median, and whiskers extended to the 5th and 95th percentiles.

were defined from the 7 CASA kinematic parameters. Sperm populations have previously been identified in fresh boar ejaculates (Abaigar et al, 1999; Quintero-Moreno et al, 2004). Since either the CASA system or the kinematic parameters used to define the sperm populations in these studies were different from the one used in the present study, it is difficult to compare the movement patterns of the sperm populations identified. In spite of this, kinematics as well as distribution rates of the motile spermatozoa identified by Abaigar et al (1999) in fresh, motile spermatozoa, using the Hobson Sperm Tracker, and the same multivariate pattern analysis that was used by us were similar to those in the present experiment. The largest sperm population (P1) showing progressive and/or vigorous movement was numerically followed by P2, spermatozoa that were moderately vigorous but poorly progressive, probably due to low cyclic adenosine monophosphate levels (Holt and Harrison, 2002). Finally these 2 populations were followed by the nonprogressive spermatozoa of P3, the smallest population. Structural alterations or major biochemical changes affected spermatozoa from P3. Poorly motile spermatozoa, such as those from P2 and P3, are less likely to progress within particular areas of the female reproductive tract (eg, the oviduct) and are therefore less likely to be involved in fertilization.

As would be expected, the 3 sperm populations identified were present throughout all 5 steps of the cryopreservation procedure. However, it is surprising that their frequency of distribution remained constant, with P1 always the most representative, containing 80% to 90% of the motile spermatozoa. Together, these results could suggest that the cryopreservation procedure does not generally affect the overall pattern of movement in most motile boar spermatozoa. This is a somewhat drastic conclusion, since frozen-thawed boar spermatozoa have more difficulties than their fresh counterparts in making progress in the sow genital tract and in fertilization of the oocytes after AI (Waberski et al, 1994). Therefore, although it seems obvious that P2 and P3 spermatozoa are less likely to successfully progress within the sow's oviduct, it also seems clear that not all of the spermatozoa of P1 are able to reach the oocytes, at least for thawed spermatozoa. In this context, a simple application of a multivariate pattern analysis to allocate motile spermatozoa into populations on the basis of their kinematic parameters does not seem to be fully informative.

The wide range of percentiles observed for most of the kinematic parameters within P1 indicated a high variability with respect to sperm movement patterns. This led us to investigate whether there was a proportion of motile spermatozoa from P1 that could have a movement pattern suitable for the estimation of fertilizing capacity. Therefore, a second multivariate pattern cluster analysis was carried out by PATN with the P1 spermatozoa. Three different sperm subpopulations were then identified. sP1, the largest, included those spermatozoa that were highly progressive with significant lateral head movement. sP2, the smallest, included moderately progressive but not very

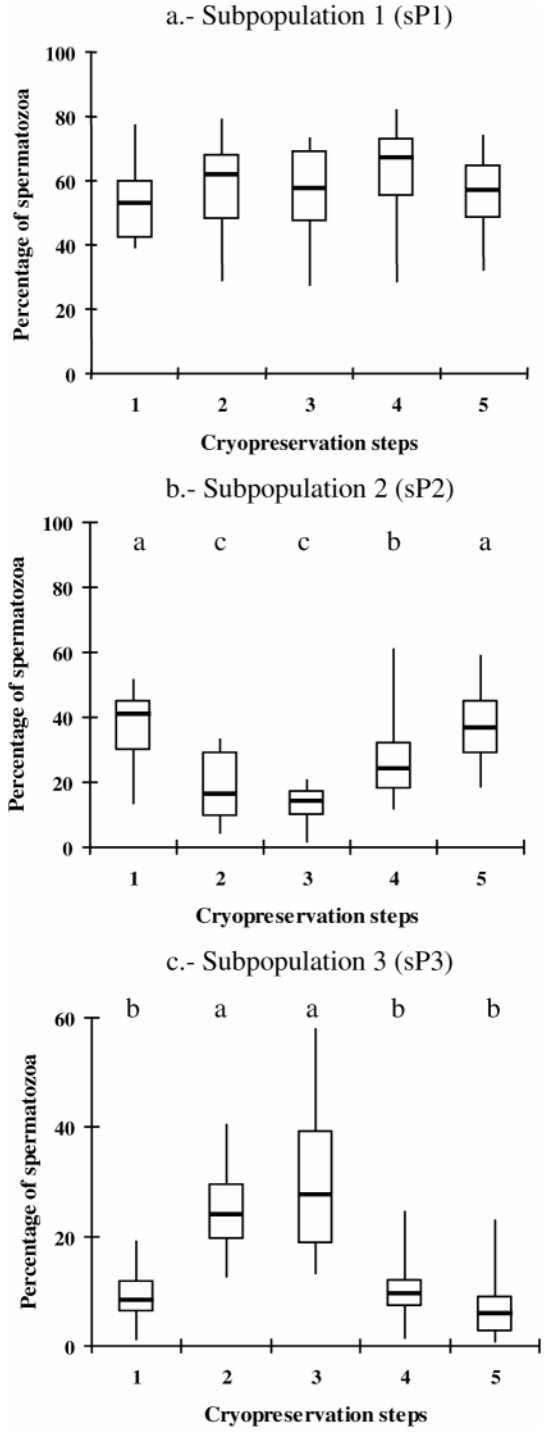

Figure 3. Whisker-box plots showing frequency distribution for the 3 sperm subpopulations (sP1–sP3) distinguished by pattern analysis within the motile spermatozoa of population 1 (P1) throughout 5 cryopreservation steps. Boxes enclose the 25th and 75th percentiles, the dot is the median, and whiskers extend to the 5th and 95th percentiles. **(a–c)** indicate significant differences ($P < .05$) in the frequency distribution among cryopreservation steps.

vigorous spermatozoa, while sP3 included spermatozoa that were very vigorous but nonprogressive. After the second cluster analysis, it became evident that P1 was not a homogeneous population.

Because selection of ''competent'' spermatozoa occurs during sperm transport in a sow's genital tract, it is reasonable to presume that sP1 spermatozoa exhibited a pattern of movement that could be related to fertility. Since the frequency of distribution of this subpopulation (sP1) did not vary through the cryopreservation procedure, between 45% and 55% of the total motile spermatozoa present in the 5 steps of the cryopreservation procedure have the kinematic ability to progress in the genital tract of the sow. This finding, which may have limited relevance for fresh or cooled semen (motile spermatozoa constitute >80% of the sample), appears relevant for thawed spermatozoa where motile spermatozoa seldom constitute 50% of the sample (Roca et al, 2003). Following the same reasoning, only about 25% of the thawed spermatozoa present in an AI dose may have the capability to progress toward fertilization. This would explain, at least in part, the poor fertility potential of frozen-thawed boar spermatozoa after AI and the relative improvement that is observed when a twofold or threefold increase in frozen-thawed sperm numbers is used.

Moreover, the proportions of spermatozoa with progressive and vigorous movement for thawed semen samples varied significantly among ejaculates (eg, boars). Such variations might account, at least in part, for the variability in fertility (in vivo or in vitro) reported elsewhere among ejaculates and boars when using frozen-thawed semen. Boars can, given on their postthaw semen quality, be classified as ''good'' or ''bad'' freezers (Thurston et al, 1999). However, definition as a ''good'' freezer is no guarantee of a high fertilizing ability for its thawed spermatozoa, since not all those called ''good'' freezers have been able to achieve high rates of in vitro fertility. Moreover, some so-called ''bad'' freezers could have such an ability (Gil et al, 2005). Therefore, as postthaw survival is an expression of sperm heterogeneity, it is possible that particular features of this heterogeneous sperm population, such as patterns of motility, can better explain the potential fertilizing ability of thawed semen than the overall percentage of postthaw motile spermatozoa. Thus, the use of 2 consecutive cluster analyses to identify the relative size of the sperm subpopulation with progressive and vigorous motility (sP1) present in thawed semen is a feature to consider. However, the implications that the different motile sperm populations or subpopulations and their distribution on thawed semen can have on its subsequent in vivo or in vitro fertility require further investigation.

Knowledge of the changes in sperm motility patterns through cryopreservation can provide important infor-

mation for understanding the physiological events that spermatozoa undergo during this handling. As an example, the peculiar pattern of movement shown by those spermatozoa of the sP3, of a vigorous nonlinear movement characterized by high VCL and ALH and low LIN, drew our attention. Recently, Schmidt and Kamp (2004) defined as hyperactivated boar spermatozoa with high VCL (≥97 μm/s) and ALH (≥3.5 μm/s) and low LIN (≤35%). According to this definition, we could consider that the spermatozoa of sP3 were hyperactivated. In contrast to what happens with sP1, the frequency distribution of sP3 varied through the 5 steps of the cryopreservation procedure, being particularly high in steps 2 and 3. Step 2 coincides with the removal of seminal plasma by centrifugation at 17°C, while step 3 coincides with the holding of the re-extended sperm pellet at 5°C. In nature, hyperactivation, a change in sperm motility concomitant with physiological capacitation, should occur during sperm transit through the female reproductive tract at the fertilization time (Ho and Suarez, 2001). Thus, large numbers of spermatozoa should not exhibit hyperactivated patterns during in vitro storage. Changes in sperm movement patterns can reflect physiological events within the spermatozoon. It is probable that an increase in the percentage of hyperactivated spermatozoa at the above-mentioned steps can be related to seminal plasma removal and plasma membrane destabilization (capacitation-like changes) associated with the slow cooling process before freezing. Seminal plasma contains decapacitation factors that inhibit the development of hyperactivated motility (Mortimer et al, 1998). Slow cooling at 5°C before freezing induces capacitation-like membrane changes in boar spermatozoa (Maxwell and Johnson, 1997; Green and Watson, 2001). Considering the drastic fall in motile spermatozoa postthaw leads to the speculation that the large population of hyperactivated spermatozoa present at steps 2 and 3 would express a continuing process of membrane destabilization during freezing and thawing, ultimately leading to cell death. Although no clear evidence of this process exists, the high proportion of acrosome-reacted cells present in thawed boar semen (Roca et al, 2004) clearly links freezing and thawing as causes for these changes.

In conclusion, the different motility patterns present in the boar ejaculate can be used to define sperm populations. Such sperm populations and the motility pattern vary during the cryopreservation process. A clear increase in the proportion of hyperactivated spermatozoa was detected at 2 steps prefreezing: 1) after removing the seminal plasma at 17°C and 2) after the slow cooling at 5°C. The study also demonstrated that the proportion of spermatozoa exhibiting progressive and vigorous movement (identified by 2 consecutive cluster analyses) remained constant through the cryopreservation procedure. Although this subpopulation can represent up to half of the total of motile spermatozoa present on thawed semen, there are differences among ejaculates (boars).

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